AD-A189 486 SAFETY TESTING OF DENGUE-1 AND DENGUE-3 SEEDS FOR HUMAN 1/1
CHALLENGES UNATTENUATED(U) FLOW LABS INC MCLEAN VA
L POTASH 26 OCT 87 DAMD17-86-C-6188

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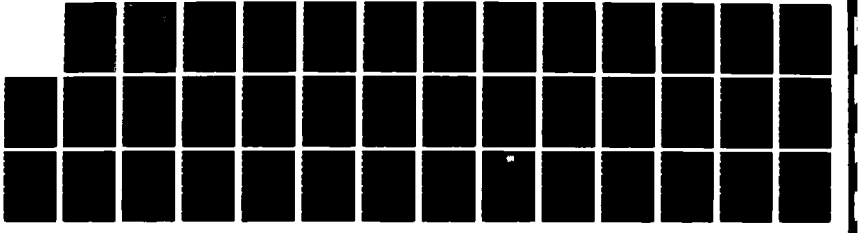

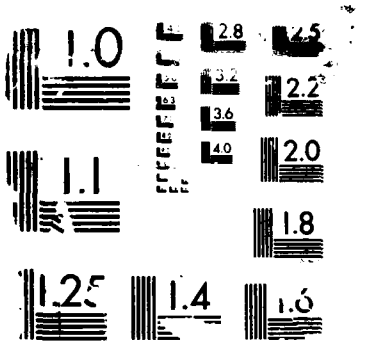
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4. PERFORMING ORGANIZATION REPORT NUMBER(S)		5. MONITORING ORGANIZATION REPORT NUMBER(S)

6a. NAME OF PERFORMING ORGANIZATION	6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
Flow Laboratories, Inc.		
6c. ADDRESS (City, State, and ZIP Code)		**7b. ADDRESS (City, State, and ZIP Code)**
McLean, Virginia 22102		

8a. NAME OF FUNDING/SPONSORING ORGANIZATION	8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
U.S. Army Medical Research & Development Command	SGRD-RMI-S	DAMD17-86-C-6188

8c. ADDRESS (City, State, and ZIP Code)	10. SOURCE OF FUNDING NUMBERS			
	PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
Fort Detrick Frederick, Maryland 21701-5012	62770A	3M1- 62770A870	AC	029

11. TITLE (Include Security Classification)

(U) Safety Testing of Dengue-1 and Dengue-3 Seeds for Human Challenges, Unattenuated

12. PERSONAL AUTHOR(S)

Potash, Louis

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Phase	FROM _____ TO _____	1987 October 26	39

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
06	13		Dengue-1; Dengue-3; Hepatitis A; Viral seeds; Vaccine
06	03		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Japanese Encephalitis (JE),Strain SA14-14-2 - Production Seed Virus Lot No. PDK8-WR2, Mfg. Date Dec 86 and Live Attenuated Virus Vaccine Lot No. PDK9-WR3, Mfg. Date Feb 87 were satisfactorily safety-tested in accordance with guidelines established by the FDA for live and inactivated vaccines as stipulated in 21 CFR, Parts 610.11, 610.12, 630.10 - 630.18, etc. All testing procedures were carried out following Good Laboratory Practices regulations (21 CFR, Part 58).

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	UNCLASSIFIED

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mary Frances Bostian	301/663-7325	SGRD-RMI-S

AD_____

SAFETY TESTING OF DENGUE-1 AND DENGUE-3 SEEDS FOR HUMAN CHALLENGE, UNATTENUATED

PHASE REPORT

LOUIS POTASH

October 26, 1987

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

CONTRACT NO. DAMD17-86-C-6188

Flow Laboratories, Inc.
McLean, Virginia 22102

Approved for public release; distribution unlimited

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the Guide for the Care and Use of Laboratory Animals prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHHS, PHS, NIH Publication No. 85-23, Revised 1985).

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I. INTRODUCTION

The accompanying protocol is a description of the safety testing of a Production seed and Vaccine of Japanese Encephalitis designated as:

Japanese Encephalitis Virus
Production Seed, Lot No. PDK8-WR2
Strain: SA14-14-2; Mfg. Date Dec 86
and
Japanese Encephalitis Vaccine
Live Attenuated Virus, Lot No. PDK9-WR3
Strain: SA14-14-2; Mfg. Date Feb 87

Utilizing the testing procedures herein described, this fluid is considered to have passed satisfactorily all tests for safety including purity. The detailed records with respect to passage history, pool production, final product, virus characterization and subsequent safety testing may be found in the laboratory notebooks located at:

The Walter Reed Army Institute of Research (WRAIR), Bldg. 501, Washington, DC 20307-5100 - (Dr. Ken Eckels)

The Experimental Virus Vaccine Production & Testing Laboratory - Suite #500 - Flow Laboratories, Inc., McLean, VA - (Dr. Louis Potash)

In conducting the tests described in this report, the investigator(s) adhered to the Good Laboratory Practices regulations (21 CFR, Part 58) and followed the guidelines established by the FDA for live and inactivated vaccines as found in 21 CFR, Parts 610.11, 610.12, 610.30, 630.10 - 630.18, etc. The procedures employed are detailed in the following SOPs and recorded on the indicated VVPL Forms:

SOP No.:	400.002 -	Issued	25 Feb 1980,	Revised	18 Feb	1986
	400.004 -	"	25 Feb 1980,	"	18 Feb	1986
	400.005 -	"	25 Feb 1980,	"	18 Feb	1986
	400.006 -	"	25 Feb 1980,	"	18 Feb	1986
	400.007 -	"	25 Feb 1980,	"	18 Feb	1986
	400.008 -	"	12 Apr 1984,	"	18 Feb	1986
	400.009 -	"	3 May 1984,	"	18 Feb	1986
	500.001 -	"	29 Oct 1980,	"	18 Feb	1986
	500.002 -	"	29 Oct 1980,	"	18 Feb	1986
	500.008 -	"	13 Jan 1981,	"	3 Mar	1986
	500.009 -	"	23 Feb 1981,	"	3 Mar	1986
VVPL FORM	#001 -	Issued	25 Feb 1981,	Revised	2 Mar	1984
	003 -	"	3 Apr 1984			
	004 -	"	16 Jan 1981,	"	21 Mar	1984
	008 -	"	29 Oct 1980,	"	3 May	1984
	015 -	"	15 Jan 1981,	"	13 July	1984
	016 -	"	15 Jan 1981,	"	13 July	1984
	017 -	"	16 Jan 1981,	"	13 Jan	1986
	023 -	"	19 Feb 1986			

II. SYNOPSIS

A. Virus Strain: Japanese Encephalitis Virus Strain: SA14-14-2

B. Live Virus Pool Designations: Production Seed, Lot No. PDK8-WR2
Mfg. Date: Dec 86
Attenuated Vaccine, Lot No. PDK9-WR3
Mfg. Date: Feb 87

C. Treatment/Handling: Freeze-Dried Fluids: Rehydrated to 6 ml with Sterile Distilled Water

D. Safety Tests on Crude Harvest Fluids:

1. Sterility: Fluid Thioglycollate (FTM), Tryptone Soya Broth (TSB), Lowenstein-Jensen Egg Medium, Mycoplasma

a.	Production Seed Virus Fluid	(52 ml)	No Growth
b.	Production Seed Control Fluid	(52 ml)	No Growth
c.	Vaccine Virus Fluid	(52 ml)	No Growth
d.	Vaccine Control Fluid	(52 ml)	No Growth

2. Tissue Culture Identity and Purity (Safety): AGMK, PHA, PDK, PRK, and Flow 5000.

a.	Production Seed Virus Fluid	(77 ml)	Satisfactory
b.	Production Seed Control Fluid	(75 ml)	Satisfactory
c.	Vaccine Virus Fluid	(77 ml)	Satisfactory
d.	Vaccine Control Fluid	(75 ml)	Satisfactory

3. Animal Safety:

a. Adult Mice: Intracerebral and I.P.

(1)	Production Seed Fluids		
	Virus Neutralized	(11 ml)	Satisfactory
	Virus Un-neutralized	(11 ml)	Satisfactory
	Control Fluid	(11 ml)	Satisfactory
(2)	Vaccine Fluids		
	Virus Neutralized	(11 ml)	Satisfactory
	Virus Un-neutralized	(11 ml)	Satisfactory
	Control Fluid	(11 ml)	Satisfactory

b. Suckling Mice: Intracerebral and I.P.

(1)	Production Seed Fluids		
	Virus Neutralized	(2.5 ml)	Satisfactory
	Virus Un-neutralized	(2.5 ml)	All died
	Control Fluid	(2.5 ml)	Satisfactory
(2)	Vaccine Fluids		
	Virus Neutralized	(2.5 ml)	Satisfactory
	Virus Un-neutralized	(2.5 ml)	All died
	Control Fluid	(2.5 ml)	Satisfactory

3. Animal Safety (continued):

c. Guinea Pigs: Intracerebral and I.P.

(1)	Production Seed Fluids		
	Virus Neutralized	(15.5 ml)	Satisfactory
	Virus Un-neutralized	(15.5 ml)	Satisfactory
	Control Fluid	(15.5 ml)	Satisfactory
(2)	Vaccine Fluids		
	Virus Neutralized	(15.5 ml)	Satisfactory
	Virus Un-neutralized	(15.5 ml)	Satisfactory
	Control Fluid	(15.5 ml)	Satisfactory

d. Rabbits: Intradermal, Subcutaneous and Corneal

(1)	Production Seed Fluids		
	Virus Fluid	(20 ml)	Satisfactory
	Control Fluid	(20 ml)	Satisfactory
(2)	Vaccine Fluids		
	Virus Fluid	(20 ml)	Satisfactory
	Control Fluid	(20 ml)	Satisfactory

e. Embryonated Eggs

(1)	Allantoic Route			
	(a)	Production Seed Fluids		
		Virus Fluid	(5 ml)	No Hemagglutination
		Control Fluid	(5 ml)	No Hemagglutination
	(b)	Vaccine Fluids		
		Virus Fluid	(5 ml)	No Hemagglutination
		Control Fluid	(5 ml)	No Hemagglutination
(2)	Yolk Sac Route			
	(a)	Production Seed Fluids		
		Virus Fluid	(5 ml)	Viability Confirmed
		Control Fluid	(5 ml)	Viability Confirmed
	(b)	Vaccine Fluids		
		Virus Fluid	(5 ml)	Viability Confirmed
		Control Fluid	(5 ml)	Viability Confirmed

E. Final Product Testing:

1. Microbial Sterility: Fluid Thioglycollate & Soybean-Casein Digest Media

a.	Production Seed Virus	(18 x 6 ml vials)	No Growth
b.	Vaccine Virus	(20 x 6 ml vials)	No Growth

2. Reverse Transcriptase:

a.	Production Seed Virus	(2 ml)	No RT Enzyme
b.	Production Seed Control	(2 ml)	No RT Enzyme
c.	Vaccine Virus	(2 ml)	No RT Enzyme
d.	Vaccine Control	(2 ml)	No RT Enzyme

E. Final Product Testing (continued):

3. General Safety:

a. Mice: I.P.

Production Seed Virus	(2 x 0.5 ml)	Satisfactory
Vaccine Virus	(2 x 0.5 ml)	Satisfactory

b. Guinea Pigs: I.P.

Production Seed Virus	(2 x 5.0 ml)	Satisfactory
Vaccine Virus	(2 x 5.0 ml)	Satisfactory

III. DETAILED SUMMARY RELATING TO THE SAFETY TESTING OF A JAPANESE ENCEPHALITIS (JE) PRODUCTION SEED AND JAPANESE ENCEPHALITIS (JE) VACCINE, PROPAGATED IN PRIMARY DOG KIDNEY CELL CULTURES

A. Inocula

On April 16, 1987, the following frozen materials were obtained for testing from Dr. K. Eckels, Contracting Officer's Representative, at the Walter Reed Army Institute of Research (WRAIR), Bldg. 501, Washington, DC 20307-5100.

1. Control Fluids (JE production seed PDK-8 - WR2) unclarified of 13 Dec 1986: 10 x 25 ml vials

2. Virus Fluids (JE production seed (PDK-8 - WR2) unclarified of 13 Dec 1986: 12 x 25 ml vials

3. Control Fluids (JE vaccine PDK-9 - WR3) unclarified of 12 Feb 1987: 10 x 25 ml vials

4. Virus Fluids (JE vaccine PDK-9 - WR3) unclarified of 12 Feb 1987: 12 x 25 ml vials

5. JE production seed PDK-8 - WR2 (freeze-dried): 21 x 6 ml vials

6. JE vaccine PDK-9 - WR3 (freeze dried): 23 x 6 ml vials

7. Rabbit Antiserum: JE 14 Parent smb 3 - Day 31 post

On arrival in this laboratory, the materials were stored as follows: Items #1 - #4 at -70°C, or below; Items #5 - #7 at -20°C, or below.

B. Safety Testing Procedures and Results on the Crude, Unclarified Harvest Fluids (SOP No.: 500.008)

1. Microbial Sterility - (VVPL FORM #019)

Aliquots of the bulk frozen fluids were thawed and tested for microbial sterility as follows:

a. Fluid Thioglycollate Medium - FTM - (LOT #35045210A): Each of 4 groups of 10 culture tubes (9-10 ml medium per tube) was inoculated with 1 ml volumes of either the crude virus fluids or the crude control fluids. An additional 10 cultures were included as uninoculated controls. All cultures were vortex mixed and incubated at 31°C (± 1°C) for 21 days with periodic examination for evidence of growth. No growth was observed in any of the 50 culture tubes.

b. Trytone Soya Broth - TSB - (LOT #35060225): Each of 4 groups of 10 culture tubes (9-10 ml medium per tube) was inoculated with 1 ml volumes of either the crude virus fluids or the crude control fluids. An additional 10 cultures were included as uninoculated controls. All cultures were vortex mixed and incubated at 22°C (± 2°C) for 21 days with periodic examination for evidence of growth. No growth was observed in any of the 50 culture tubes.

c. Lowenstein-Jensen Egg Medium (DIFCO - Lot #752211): Each of 4 groups of 10 slant culture tubes was inoculated with 0.5 ml of either the crude virus fluids or the crude control fluids. Ten additional culture tubes were included as uninoculated controls. All cultures were incubated at 36.5°C (± 1°C) - horizontally for the first 24 hours and then vertically for the remainder of the 8-week observation period. Cultures were examined periodically for growth over this 8-week period. No growth was observed in any of the 50 slant culture tubes.

The results of the above described microbial sterility assays are summarized in Table I.

d. Mycoplasma Sterility: These assays were performed by Flow Laboratories' Mycoplasma Testing Laboratory and included both the routine PPLO agar and broth assays and the specific test for the detection of M. hyorhinis. Samples (1 x 25 ml and 1 x 2 ml) of the two crude virus and two crude control fluids were submitted for testing. The samples were reported to be negative for mycoplasmas. Copies of these reports are appended to this Protocol - (Appendixes - 1, 2 and 3).

2. *Identity in Tissue Culture (Serum-Neutralization)* - (VVPL FORM #015)

An attempt to identity the crude virus pool was carried out using AGMK tube cultures. Equal volumes of the crude vaccine virus pool and a 1:3 dilution of the rabbit immune serum were mixed and incubated at 35°C (± 1°C) for 2 hours. To each of 4 tissue culture tubes was added 0.4 ml of the serum-virus mixture. In addition, to each of 2-4 tubes was added 0.2 ml of either the undiluted crude vaccine virus fluid or the diluted immune serum. Four culture tubes were included as uninoculated cell lot controls. Prior to inoculation tube cultures were refed with 2 ml of Medium MEM containing 5% fetal bovine serum (heat inactivated) plus antibiotics - (VVPL-MM-187-3). Cultures were incubated at 35°C for 7 days at which time some morphological changes were detected only in the virus control cultures. Cultures were tested for hemadsorption - medium was decanted and 1 ml of 0.1% guinea pig RBC (in PBS) was added per tube with incubation at 4°C for a minimum of 30 min. Films were examined microscopically for hemadsorption - all were negative.

3. Purity (Safety) in Tissue Cultures - (VVPL FORM #016)

a. Tissue Cultures: Fully "sheeted" flask or roller tube cell cultures were prepard by laboratory personnel. Cultures were maintained on Medium MEM containing 2 to 10% fetal bovine serum (heat-inactivated) plus antibiotics (in mcg/ml): gentamicin, 100; neomycin, 50; and amphotericin B (I.V.), 2.5. Cultures were inoculated, refed and subpassaged as indicated below. The following tissue culture systems were utilized:

(1)	Tertiary African Green Monkey Kidney (AGMK)	MEM + 5% serum
(2)	Primary Human Amnion (PHA)	MEM + 10% serum
(3)	Primary Dog Kidney (PDK)	MEM + 5% serum
(4)	Primary Rabbit Kidney (PRK)	MEM + 5% serum
(5)	Whole Human Embryo Fibroblast (Flow 5000)	MEM + 5% serum

b. General Testing Procedures

(1) Crude Virus Fluids

(a) Primary Flask Cultures: Equal volumes of the bulk crude virus fluids (production seed and vaccine) and a 1:3 dilution of the rabbit immune serum were well mixed and incubated at 35°C (± 1°C) for 2 hours. A total of 15 ml of each of the virus fluids was tested per tissue culture system where-in each of 2 - 75 cm^2 flasks per tissue culture system was inoculated with 15 ml of these serum-virus mixture. Flasks contained approximately 25 ml of maintenance medium at the time of inoculation. Cultures were incubated at 35°C (37°C for PHA) for 14 days with periodic microscopic examination for any signs of CPE and/or cellular degradation. When necessary to maintain the integrity of the cell films, cultures were refed with 35 ml of fresh medium.

(b) Secondary Tube Subcultures: On the 14th day of incubation, the primary cultures were re-examined microscopically and the fluids harvested individually and treated with the specific immune serum - 0.1 ml per harvest. In addition, to each individual harvest was added: 0.1 ml gentamicin (50 mg/ml); 1 ml penicillin-streptomycin solution (5000 units/ml and 5000 mcg/ml, respectively); and 10% of 10X SPG* (v/v). Following mixing, the fluids were incubated at room temperature for 60 minutes and then subpassed into homologous roller tube cultures - 0.5 ml of each harvest into each of 20 tubes. The remainder of the harvest fluids was saved and stored at -75°C, or below. All primary cultures were tested for hemadsorption by the addition of 0.1% guinea pig RBC (in PBS) and incubation at 4°C for a minimum of 30 minutes. All cultures were negative for hemadsorption.

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* 10X SPG: sucrose, 2.18 M; KH_2PO_4, 0.038 M; K_2HPO_4, 0.072 M; potassium glutamate, 0.049 M.

Tube cultures (refed with 2 ml of maintenance medium prior to inoculation) were incubated at 35°C (37°C for PHA) for 14 additional days. When necessary to maintain the integrity of the cell films, cultures were refed with 2 ml of fresh medium. Cultures were examined microscopically at periodic intervals and at the end of the incubation period for any signs of CPE. After final examination, tubes were divided - depending on the specific cell system - for additional testing:

AGMK, PHA, and Flow 5000 Tube Cultures: These were divided into 3 groups as follows:

1/4th tested for the presence of hemadsorbing agents,
1/4th fixed and stained with a solution of 5% glutaraldehyde + 1:10 giemsa stain and examined microscopically for any CPE,
1/2 challenged with Coxsackie A-9 virus (0.2 ml per each of 2 tubes at the dilutions noted in the tables) for the detection of non-CPE producing agents and/or latent agents via the interference phenomenon.

PDK and PRK Tube Cultures: These were equally divided into 2 groups:

1/2 tested for the presence of hemadsorbing agents,
1/2 fixed and stained with the glutaraldehyde-giemsa stain solution and examined microscopically for any CPE.

No challenge studies were carried out with the Coxsackie A-9 virus since this virus does not produce any discernible CPE in these tissue culture systems.

(2) Crude Control Fluids

Equal volumes of the two crude control fluids (production seed and vaccine) and the indicated maintenance medium were well mixed and incubated at 35°C for 2 hours. A total of 15 ml of each of the control fluids was tested per tissue culture system wherein each of 2 - 75 cm^2 flasks per tissue culture system was inoculated with 15 ml of the above mixtures. Cultures were handled in a manner similar to that described above for the crude virus fluids except that immune serum was not included.

(3) Uninoculated Cell Lot Controls

Two 75-cm^2 flasks or bottles per tissue culture system were included as uninoculated cell lot controls and were handled in a manner similar to that described above for the crude virus fluids except that immune serum was not included. In addition, an appropriate number of uninoculated roller tube cultures were included as cell lot controls for the secondary tube subcultures.

In all challenge studies, 1 to 4 culture tubes per set were left unchallenged to serve as controls to the challenge virus.

The results of these in vitro Tissue Culture Purity (Safety) tests are summarized in Tables II-A through -E.

4. Animal Safety Tests - (VVPL FORM #004)

a. Adult Mice - Test for adventitious agents - (SOP No. 400.005)

For these studies, adult CD-1 mice (15-20 grams each) were used with the indicated crude fluids inoculated intracerebrally with 0.03 ml and intraperitoneally with 0.5 ml.

(1) Production Seed Fluids: Each of 3 groups of 20 mice was inoculated with either the crude un-neutralized virus fluid, the crude neutralized* virus fluid or the crude control fluid. An additional 10 mice were included as uninoculated controls.

(2) Vaccine Fluids: Each of 3 groups of 20 mice was inoculated with either the crude un-neutralized virus fluid, the crude neutralized* virus fluid or the crude control fluid. An additional 5 mice were included as uninoculated controls.

The mice were observed daily for deaths and/or signs of illness or distress over a 4 week period. All mice (inoculated as well as controls) remained healthy and survived the entire 28-day observation period with no evidence of lymphocytic choriomeningitis virus infection or of any other virus infection. This test in adult mice was considered satisfactory.

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* Only the intracerebral inoculum was neutralized at the rate of 0.2 ml of undiluted immune rabbit serum to 0.6 ml of crude virus fluid with incubation at 35°C (± 1°C) *for 2 hours.*

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b. Suckling Mice - Test for adventitious agents - (SOP No.: 400.005)

For these studies, newborn CD-1 mice from mixed litters (10 per mother - less than 24 hours old) were used with the indicated crude fluids inoculated intracerebrally with 0.01 ml and intraperitoneally with 0.1 ml.

(1) Production Seed Fluids: Each of 3 groups of 20 sucklings was inoculated with either the crude un-neutralized virus fluid, the crude neutralized* virus fluid or the crude control fluid. An additional litter of 10 sucklings was included as uninoculated controls. All sucklings were observed daily for 14 days for deaths and/or signs of illness or distress. By day 9, all 20 sucklings inoculated with the un-neutralized crude virus were found cannibalized. On day 10, 2 of the sucklings inoculated with the crude neutralized virus were found cannibalized. There were no other deaths and none of the sucklings (other than those inoculated with the un-neutralized virus) exhibited any signs of illness or distress over this initial 14-day observation period.

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* Both the intracerebral and intraperitoneal inocula were neutralized at the rate of 0.5 ml of undiluted immune rabbit serum to 2.0 ml of crude virus fluid with incubation at 35°C (± 1°C) for 2 hours.

On the 14th day, single pools were prepared of the emulsified tissue (minus skin and viscera) of the following groups: a) neutralized virus inoculated sucklings (18); b) control fluid inoculated sucklings (20); and c) uninoculated controls (10). A blind passage into mixed litters of newborn CD-1 mice was made of each of the 3 pools via the intracerebral and intraperitoneal routes: the individual pools from the inoculated sucklings (a and b) into each of 20 newborns and the pool from the uninoculated control sucklings (c) into 10 newborns. An additional litter of 10 sucklings was included as uninoculated controls (d) for this blind passage. All sucklings were observed daily for 14 days for deaths and/or signs of illness or distress. Of the sucklings inoculated with pool 'c' (originally uninoculated controls), three (3) were found cannibalized within the first 24 hours. There were no other deaths and none of the sucklings exhibited any signs of illness or distress over this final 14-day observation period.

Since none of the inoculated sucklings (neutralized virus or control fluid) exhibited any evidence of a transmissible agent or of Coxsackie virus infection or of any viral infection, and since at least 90% of the these inoculated sucklings remained healthy and survived the entire observation period, this test with production seed fluids in suckling mice was considered satisfactory.

(2) Vaccine Fluids: Each of 3 groups of 20 sucklings was inoculated with either the crude un-neutralized virus fluid, the crude neutralized* virus fluid or the crude control fluid. An additional litter of 10 suclklings was included as uninoculated controls. All sucklings were observed daily for 14 days for deaths and/or signs of illness or distress. By day 8, all 20 sucklings inoculated with the un-neutralized crude virus were found cannibalized. On day 14, one (1) of the sucklings inoculated with the crude control fluid was found cannibalized without having exhibited any prior signs of illness or distress. There were no other deaths and none of the sucklings (other than those inoculated with the un-neutralized virus) exhibited any signs of illness or distress over this initial 14-day observation period.

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* Both the intracerebral and intraperitoneal inocula were neutralized at the rate of 0.5 ml of undiluted immune rabbit serum to 2.0 ml of crude virus fluid with incubation at 35°C (± 1°C) for 2 hours.

On the 14th day, single pools were prepared of the emulsified tissue (minus skin and viscera) of the following groups: a) neutralized virus inoculated sucklings (20); b) control fluid inoculated sucklings (19); and c) uninoculated controls (10). A blind passage into mixed litters of newborn CD-1 mice was made of each of the 3 pools via the intracerebral and intraperitoneal routes: the individual pools from the inoculated sucklings (a and b) into each of 20 newborns and the pool from the uninoculated control sucklings (c) into 10 newborns. An additional litter of 10 sucklings was included as uninoculated controls (d) for this blind passage. All sucklings were observed daily for 14 days for deaths and/or signs of illness or distress. Within the first 24 hours, a total of 5 sucklings were found cannibalized, 3 from sucklings inoculated with pool (a) and 2 from sucklings inoculated with pool (c). There were no other deaths and none of the sucklings exhibited any signs of illness or distress over this final 14-day observation period.

Since none of the inoculated sucklings (neutralized virus or control fluid) exhibited any evidence of a transmissible agent or of Coxsackie virus infection or of any viral infection, and since at least 90% of the these inoculated sucklings remained healthy and survived the entire observation period, this test with vaccine fluids in suckling mice was considered satisfactory.

c. Adult Guinea Pigs - (SOP No.: 400.006)

Test for M. tuberculosis: For these studies, adult guinea pigs (Hartley Strain, virus free, 350-450 grams each) were used with the indicated crude fluids inoculated intracerebrally with 0.1 ml and intraperitoneally with 5.0 ml.

(1) Production Seed Fluids: Each of 3 groups of 3 guinea pigs was inoculated with either the crude un-neutralized virus fluid, the crude neutralized* virus fluid or the crude control fluid. An additional group of 3 guinea pigs was included as uninoculated controls. All pigs were observed daily for a period of 6 weeks for deaths and/or any signs of illness or distress. There were no reported or recorded deaths or signs of illness or distress. Commencing on day 21, daily rectal temperatures (LED digital thermistor thermometer) were taken and recorded (± 0800 hrs) for all guinea pigs until time of sacrifice. The average temperatures (^{o}C) for the 4 groups of guinea pigs were:

a) un-neutralized virus fluid - 38.33, 38.48 and 38.52;
b) neutralized virus fluid - 38.44, 38.50 and 38.51;
c) control fluid - 38.48, 38.52 and 38.61;
d) uninoculated controls - 38.32, 38.42 and 38.47.

There were no significant rises indicative of either bacterial or viral infection. All guinea pigs appeared healthy and survived the entire 42-day observation period at which time they were necropsied following euthanasia with Halathane. Inspection of the abdominal and thoracic cavities indicated no gross pathological changes. This test in guinea pigs with the production seed fluids was considered satisfactory.

(2) Vaccine Fluids: Each of 3 groups of 3 guinea pigs was inoculated with either the crude un-neutralized virus fluid, the crude neutralized* virus fluid or the crude control fluid. An additional group of 3 guinea pigs was included as uninoculated controls. All pigs were observed daily for a period of 6 weeks for deaths and/or any signs of illness or distress. There were no reported or recorded deaths or signs of illness or distress. Commencing on day 21, daily rectal temperatures (LED digital thermistor thermometer) were taken and recorded (± 0800 hrs) for all guinea pigs until time of sacrifice. The average temperatures (^{o}C) for the 4 groups of guinea pigs were:

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* Only the intracerebral inoculum was neutralized at the rate of 0.1 ml of undiluted immune rabbit serum to 0.4 ml of crude virus fluid with incubation at 35^{o}C (± 1^{o}C) for 2 hours.

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a) un-neutralized virus fluid - 38.35, 38.40 and 38.53;
b) neutralized virus fluid - 38.25, 38.30 and 38.54;
c) control fluid - 38.34, 38.35 and 38.36;
d) uninoculated controls - 38.32, 38.37 and 38.45.

There were no significant rises indicative of either bacterial or viral infection. All guinea pigs appeared healthy and survived the entire 42-day observation period at which time they were necropsied following euthanasia with Halathane. Inspection of the abdominal and thoracic cavities indicated no gross pathological changes. This test in guinea pigs with the vaccine fluids was considered satisfactory.

d. Adult Rabbits - Test for B-virus and other adventitious agents - (SOP No.: 400.004)

(1) Production Seed Fluids: Each of two New Zealand white rabbits (1500-2500 grams each) was inoculated intradermally in multiple sites with a total of 1.0 ml and subcutaneously with 9.0 ml with the un-neutralized crude virus fluid. In addition, the left cornea was scratched and 0.03 ml of the virus fluid was applied. Two rabbits were similarly inoculated with the crude control fluid but with the right cornea scratched. One additional rabbit was included as an uninoculated control. All rabbits were observed daily for a total of 28 days for deaths and/or signs of lesions at sites of inoculation and for any signs of illness or distress. All rabbits remained healthy and none exhibited any signs of illness or distress or lesions at the sites of inoculation over the 4-week observation period. This test in adult rabbits with the production seed fluids was considered satisfactory.

(2) Vaccine Fluids: Each of two New Zealand white rabbits (1500-2500 grams each) was inoculated intradermally in multiple sites with a total of 1.0 ml and subcutaneously with 9.0 ml with the un-neutralized crude virus fluid. In addition, the left cornea was scratched and 0.03 ml of the virus fluid was applied. Two rabbits were similarly inoculated with the crude control fluid but with the right cornea scratched. One additional rabbit was included as an uninoculated control. All rabbits were observed daily for a total of 28 days for deaths and/or signs of lesions at sites of inoculation and for any signs of illness or distress. All rabbits remained healthy and none exhibited any signs of illness or distress or lesions at the sites of inoculation over the 4-week observation period. This test in adult rabbits with the vaccine fluids was considered satisfactory.

The results of these in vivo Animal Safety Tests are summarized in Table III - A through - D.

e. Embryonated Eggs

For these studies, only SPF-COFAL negative embryonated eggs obtained from SPAFAS, Inc. (Norwich, CT) were employed. These eggs were designated as M-95D and a copy of the Quality Control Sheet is appended to this Protocol - (Appendix - 4).

(1) Allantoic Fluid Inoculation

(a) Production Seed Fluids: Each of 2 groups of ten 10-day-old embryonated eggs was inoculated via the allantoic route with 0.5 ml of either the crude un-neutralized virus or the crude control fluid. Eggs were incubated at 35°C (± 1°C) for 72 hours together with 10 uninoculated control eggs. After 72 hours, eggs were candled (none found dead), chilled at 4°C and then individually harvested. Fluids were incubated in a 37°C water bath for 60 minutes to elute any adsorbed agent and then clarified by centrifugation at 900 x g for 10 minutes. Sample pools were prepared and tested for hemagglutination with both guinea pig (0.6%) and chick (0.4%) erythrocytes (in PBS) at 4°C and at room temperature (18-21°C). All 3 sample pools were negative for hemagglutination when tested both at undiluted and at a 1:10 dilution.

The three sample pools - [a) virus inoculated, b) control inoculated and c) uninoculated] - were subpassaged into each of ten 10-day-old embryonated eggs using the same route and volume. Eggs were incubated at 35°C (± 1°C) for 72 hours together with 10 uninoculated control eggs. After 72 hours, eggs were candled with one embryo found dead from pool (a) and one from pool (c). The live eggs were chilled overnight at 4°C and then individually harvested. Fluids were handled as described above including tests for hemagglutination. All 4 pools were negative for hemagglutination when tested both at undiluted and at a 1:10 dilution.

Since none of the harvest fluids exhibited any hemaggluutination when tested against guinea pig and chick RBC, this allantoic inoculation aspect of the embryonated egg study with production seed fluids was considered satisfactory.

(b) Vaccine Fluids: Each of 2 groups of ten 10-day-old embryonated eggs was inoculated via the allantoic route with 0.5 ml of either the crude un-neutralized virus or the crude control fluid. Eggs were incubated at 35°C (± 1°C) for 72 hours together with 10 uninoculated control eggs. After 72 hours, eggs were candled with one virus inoculated embryo found dead. The live eggs were chilled at 4°C and then individually harvested. Fluids were incubated in a 37°C water bath for 60 minutes to elute any adsorbed agent and then clarified by centrifugation at 900 x g for 10 minutes. Sample pools were prepared and tested for hemagglutination with both guinea pig (0.6%) and chick (0.4%) erythrocytes (in PBS) at 4°C and at room temperature (18-21°C). All 3 sample pools were negative for hemagglutination when tested both at undiluted and at a 1:10 dilution.

The three sample pools - [a) virus inoculated, b) control inoculated and c) uninoculated] - were subpassaged into each of ten 10-day-old embryonated eggs using the same route and volume. Eggs were incubated at 35°C (± 1°C) for 72 hours together with 10 uninoculated control eggs. After 72 hours, eggs were candled with none found dead. The live eggs were chilled overnight at 4°C and then individually harvested. Fluids were handled as described above including tests for hemagglutination. All 4 pools were negative for hemagglutination when tested both at undiluted and at a 1:10 dilution.

Since none of the harvest fluids exhibited any hemaggluutination when tested against guinea pig and chick RBC, this allantoic inoculation aspect of the embryonated egg study with vaccine fluids was considered satisfactory.

(2) Yolk Sac Inoculation

(a) Production Seed Fluids: Each of 2 groups of ten 6-day-old embryonated eggs was inoculated into the yolk sac with 0.5 ml of either the crude un-neutralized virus or the crude control fluid. Eggs were incubated at 35°C (± 1°C) for 9 days together with 10 uninoculated control eggs. Eggs were candled periodically over this 9-day period with 4 deaths recorded - 2 from the virus inoculated group with one each on days 6 and 7, and 2 from the control fluid inoculated group with both on day 6. The live eggs were chilled at 4°C and the yolk sacs individually harvested, pooled and 10% suspensions in Medium MEM prepared.

The 3 sample pool suspensions - [a) virus inoculated, b) control fluid inoculated and c) uninoculated] - were subpassaged by the same route and volume into 10 fresh 6-day-old embryonated eggs. Eggs were incubated at 35°C (± 1°C) for 9 days together with 10 uninoculated control eggs. Eggs were candled periodically over this 9-day period with 2 deaths recorded - both from pool (a) with one each on days 4 (egg shell cracked on day 0) and 7.

Although there were some sporadic deaths recorded, viability for at least 80% of the inoculated eggs was confirmed. This yolk sac inoculation aspect of the embryonated egg study with production seed fluids was considered satisfactory.

(b) Vaccine Fluids: Each of 2 groups of ten 6-day-old embryonated eggs was inoculated into the yolk sac with 0.5 ml of either the crude un-neutralized virus or the crude control fluid. Eggs were incubated at 35°C (± 1°C) for 9 days together with 10 uninoculated control eggs. Eggs were candled periodically over this 9-day period with no deaths recorded. The live eggs were chilled at 4°C and the yolk sacs individually harvested, pooled and 10% suspensions in Medium MEM prepared.

The 3 sample pool suspensions - [a) virus inoculated, b) control fluid inoculated and c) uninoculated] - were sub-passaged by the same route and volume into 10 fresh 6-day-old embryonated eggs. Eggs were incubated at 35°C (± 1°C) for 9 days together with 10 uninoculated control eggs. Eggs were candled periodically over this 9-day period with a total of 3 deaths recorded all on day 5 - 2 from pool (c) and one from the uninoculated controls.

There were no deaths recorded for the virus or control fluid inoculated eggs. Since viability was confirmed, this yolk sac inoculation aspect of the embryonated egg study with vaccine fluids was considered satisfactory.

C. Final Product Testing and Results - (SOP No.: 500.009)

1. Microbial Sterility

A total of 18 x 6 ml vials of the freeze-dried production seed virus product and 20 x 6 ml vials of the freeze-dried virus vaccine final product were submitted to Ben-Venue Laboratories, Inc., for microbial sterility testing via the USP Membrane Filtration Method in Fluid Thioglycollate and Fluid Soybean-Casein Digest Media. No growth was reported for either product and copies of their reports are appended to this Protocol - (Appendixes - 5, 6 and 7).

2. Reverse Transcriptase - Assay for the detection of RNA-dependent DNA-polymerase activity

The assay for Reverse Transcriptase was performed by Dr. Allan Tereba at the St. Jude Children's Research Hospital, Memphis, TN. Two ml aliquots of the reconstituted freeze-dried virus fluids and 2 ml aliquots of the clarifed (centrifuged) control fluids were submitted for assay. All four samples were reported to be negative for the RT Enzyme and a copy of this report is appended to this Protocol - (Appendix - 8).

3. General Safety Test - (SOP No.: 400.002 - VVPL FORM #001)

Each of 2 groups of 2 overtly healthy CD-1 mice (less than 22 grams each) and each of 2 groups of 2 overtly healthy guinea pigs (Hartley Strain, virus free - less than 400 grams each) were inoculated intraperitoneally with 0.5 ml and 5 ml, respectively, of either the reconstituted freeze-dried production seed virus product or the virus vaccine final product. Two additional animals of each species were included as uninoculated controls. All animals were weighed prior to inoculation and on day 7 post inoculation. All animals were observed daily over this 7-day period for deaths and/or signs of illness or distress - none were noted. All animals remained healthy and all exhibited weight gains. This test was considered satisfactory. The results of these General Safety Tests are summarized in Table IV.

Table I. Microbial Sterility Test Results on the Crude Japanese Encephalitis Production Seed and Vaccine Control and Virus Fluids

Culture Medium	No.	Vol. per culture (ml)	Temperature	Date On Test	Date Off Test	Results
Fluid Thioglycollate (FTM) LOT #35045210A	10	-----	30-32°C	5/20/87	6/10/87	No Growth
Production Seed Virus	10	1.0		|	|	No Growth
Production Seed Control	10	1.0		|	|	No Growth
Vaccine Virus	10	1.0		|	|	No Growth
Vaccine Control	10	1.0		5/20/87	6/10/87	No Growth
Tryptone Soya Broth (TSB) LOT #35060225	10	-----	20-24°C	5/20/87	6/10/87	No Growth
Production Seed Virus	10	1.0		|	|	No Growth
Production Seed Control	10	1.0		|	|	No Growth
Vaccine Virus	10	1.0		|	|	No Growth
Vaccine Control	10	1.0		5/20/87	6/10/87	No Growth
Lowenstein-Jensen Egg Medium - LOT #752211	10	-----	35.5-37.5°C	5/20/87	7/15/87	No Growth
Production Seed Virus	10	0.5		|	|	No Growth
Production Seed Control	10	0.5		|	|	No Growth
Vaccine Virus	10	0.5		|	|	No Growth
Vaccine Control	10	0.5		5/20/87	7/15/87	No Growth

Table II. Tissue Culture Purity (Safety) Test Results on the Crude Japanese Encephalitis Production Seed and Vaccine Control and Virus Fluids

A. Tertiary African Green Monkey Kidney (AGMK)

	Initial Flasks			0.5 ml per tube Passage #1						
	Lot # 069 (2117 p3)			Lot # 076 (2133 p3)						
	Day 14			Day 14 + 14 = 28						
							Coxsackie A-9 Challenge*			
Material Tested	CPE	Hads	Stain	CPE	Hads	Stain	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Production Seed	**	***	**							
Virus/Serum Mixture	2/2	2/2	2/2	0/39	0/10	0/10	4/4	4/4	4/4	3/4
Control Fluid (TCF)	0/2	0/2	0/2	0/38	0/10	0/10	4/4	4/4	4/4	4/4
Vaccine	**	***	**							
Virus/Serum Mixture	2/2	2/2	2/2	0/40	0/10	0/10	4/4	4/4	4/4	2/4
Control Fluid (TCF)	0/2	0/2	0/2	0/40	0/10	0/10	4/4	4/4	4/4	3/4
Control - (1)	0/2	0/2	0/2	0/40	0/10	0/10	4/4	4/4	4/4	4/4
Control - (2)				0/60	0/12	0/12	8/8	8/8	8/8	6/8

* Coxsackie A-9 Challenge Results based on a 5-day incubation at 35°C.

** All virus/serum inoculated flasks exhibited some morphological changes in contrast to to the control fluid inoculated or to the cell lot controls.

*** All virus/serum inoculated flasks exhibited RBC clumps but no definitive hemadsorption.

Table II. Tissue Culture Purity (Safety) Test Results on the Crude Japanese Encephalitis Production Seed and Vaccine Control and Virus Fluids

B. Primary Human Amnion (PHA)

	Initial Flasks			0.5 ml per tube Passage #1						
	Lot # 071			Lot # 080						
	Day 14			Day 14 + 14 = 28						
							Coxsackie A-9 Challenge*			
Material Tested	** CPE	Hads	Stain	*** CPE	Hads	Stain	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Production Seed										
Virus/Serum Mixture	0/2	0/2	ND	0/40	0/10	0/10	4/4	4/4	4/4	4/4
Control Fluid (TCF)	0/2	0/2	ND	0/40	0/10	0/10	4/4	4/4	4/4	2/4
Vaccine										
Virus/Serum Mixture	0/2	0/2	ND	0/39	0/10	0/10	4/4	4/4	4/4	4/4
Control Fluid (TCF)	0/2	0/2	ND	0/40	0/10	0/10	4/4	4/4	4/4	3/4
Control - (1)	0/2	0/2	ND	0/39	0/10	0/10	4/4	4/4	4/4	4/4
Control - (2)				0/52	0/12	0/12	6/6	6/6	6/6	5/6

* Coxsackie A-9 Challenge Results based on a 4-day incubation at 37°C. Tubes refed with 2 ml of fresh medium prior to challenge.

** On day 8, all flasks refed with 35 ml of fresh medium. All day 14 harvests stored at -70°C (or below) for 28 days until lot of tubes became available for subpassage.

*** On day 23 (days 14 + 9), all tubes refed with 2 ml of fresh medium.

ND = not done

Table II. Tissue Culture Purity (Safety) Test Results on the Crude Japanese Encephalitis Production Seed and Vaccine Control and Virus Fluids

C. Primary Dog Kidney (PDK)

	Initial Flasks			0.5 ml per tube Passage #1		
	Lot # 071			Lot # 080		
	Day: 14			Day: 14 + 14 = 28 *		
Material Tested	CPE	Hads	Stain	CPE	Hads	Stain
Production Seed						
Virus/Serum Mixture	0/2	0/2	ND	0/40	0/20	0/20
Control Fluid (TCF)	0/2	0/2	ND	0/40	0/20	0/20
Vaccine						
Virus/Serum Mixture	0/2	0/2	ND	0/39	0/19	0/20
Control Fluid (TCF)	0/2	0/2	ND	0/37	0/18	0/19
Control - (1)	0/2	0/2	ND	0/40	0/20	0/20
Control - (2)				0/24	0/12	0/12

* On day 22 (days 14 + 8), all tubes refed with 2 ml of fresh medium.

ND = not done

Table II. Tissue Culture Purity (Safety) Test Results on the Crude Japanese Encephalitis Production Seed and Vaccine Control and Virus Fluids

D. Primary Rabbit Kidney (PRK)

	Initial Flasks			0.5 ml per tube Passage #1		
	Lot # 070			Lot # 079		
	Day: 14			Day: 14 + 14 = 28 *		
Material Tested	CPE	Hads	Stain	CPE	Hads	Stain
Production Seed						
Virus/Serum Mixture	0/2	0/2	ND	0/39	0/19	0/20
Control Fluid (TCF)	0/2	0/2	ND	0/39	0/19	0/20
Vaccine						
Virus/Serum Mixture	0/2	0/2	ND	0/40	0/20	0/20
Control Fluid (TCF)	0/2	0/2	ND	0/40	0/20	0/20
Control - (1)	0/2	0/2	ND	0/40	0/20	0/20
Control - (2)				0/24	0/12	0/12

* On day 23 (days 14 + 9), all tubes refed with 2 ml of fresh medium.

ND = not done

Table II. Tissue Culture Purity (Safety) Test Results on the Crude Japanese Encephalitis Production Seed and Vaccine Control and Virus Fluids

E. Whole Human Embryo Fibroblast (Flow 5000)

	Initial Flasks			0.5 ml per tube Passage #1						
	Lot # 068			Lot # 075						
	Day 14			Day 14 + 14 = 28						
							Coxsackie A-9 Challenge*			
Material Tested	CPE	Hads	Stain	CPE	Hads	Stain	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Production Seed Virus/Serum Mixture	0/2	2/2**	ND	0/40	0/10	0/10	4/4	4/4	3/4	2/4
Control Fluid (TCF)	0/2	0/2	ND	0/39	0/10	0/10	4/4	4/4	3/4	1/4
Vaccine Virus/Serum Mixture	0/2	2/2**	ND	0/40	0/10	0/10	4/4	4/4	4/4	0/4
Control Fluid (TCF)	0/2	0/2	ND	0/39	0/10	0/10	4/4	4/4	4/4	0/4
Control - (1)	0/2	0/2	ND	0/39	0/10	0/10	4/4	4/4	3/4	1/4
Control - (2)				0/60	0/12	0/12	8/8	8/8	8/8	3/8

* Coxsackie A-9 Challenge Results based on a 7-day incubation at 35°C.

** All virus/serum inoculated flasks exhibited RBC clumps but no definite hemadsorption.

ND = not done

Table III - A. Animal Safety Tests Results on the Crude Japanese Encephalitis Production Seed Control and Virus Fluids

Animal Species	Inoculum	Vol. (ml)	Route	No.	Lesions, Illness or Deaths over 4 to 6 Week Period	Comments
Adult Mice (15-20 grams)	Virus Pool Un-neutralized	0.03 0.50	I. Cer. I.P.	20	No deaths nor signs of illness or distress recorded.	Test Satisfactory
	Virus Pool Neutralized	0.03 0.50	I. Cer. I.P.	20		
	Control Fluid (TCF)	0.03 0.50	I. Cer. I.P.	20		
	None	—	—	10		
Suckling Mice (< 24 hours)	Virus Pool Un-neutralized	0.01 0.10	I. Cer. I.P.	20	3 sick on day 2 found cannibalized on day 3. Remaining 17 all found cannibalized day 9	
	Virus Pool Neutralized	0.01 0.10	I. Cer. I.P.	20	2 found cannibalized on day 10	
	Control Fluid (TCF)	0.01 0.10	I. Cer. I.P.	20	No other deaths nor signs of illness or distress over this initial 14-day period.	
	None	—	—	10		Test Satisfactory
	D14 Blind Passage (VP-N)	0.01 0.10	I. Cer. I.P.	20	3 of 14-Blind Passage (N) found cannibalized within first 24 hours.	90% survival of inoculated sucklings. No evidence of a transmissible agent or of any viral infection.
	D14 Blind Passage (CF-TCF)	0.01 0.10	I. Cer. I.P.	20		
	D14 Blind Passage (N)	0.01 0.10	I. Cer. I.P.	10	No other deaths nor signs of illness or distress over this final 14-day period.	
	D14 - None	—	—	10		

Table III - B. Animal Safety Tests Results on the Crude Japanese Encephalitis Production Seed Control and Virus Fluids

Animal Species	Inoculum	Vol. (ml)	Route	No.	Lesions, Illness or Deaths over 4 to 6 Week Period	Comments
Adult Guinea Pigs (350-450 gms)	Virus Pool Un-neutralized	0.10 5.00	I. Cer. I.P.	3	No deaths nor signs of illness or distress. Daily rectal temperatures taken over last 3 weeks of observation were within normal ranges.	
	Virus Pool Neutralized	0.10 5.00	I. Cer. I.P.	3		
	Control Fluid (TCF)	0.10 5.00	I. Cer. I.P.	3		
	None	——	——	3		

Code	GP#	Mean Temp. (^{o}C)	Temp. Range (^{o}C)
VP-UN-1	1	38.33	37.9 - 38.7
VP-UN-2	2	38.48	38.2 - 38.9
VP-UN-3	3	38.52	38.2 - 38.9
VP-N-1	4	38.51	38.2 - 38.7
VP-N-2	5	38.44	38.1 - 38.7
VP-N-3	6	38.50	38.2 - 38.8
TCF-1	7	38.52	38.2 - 38.8
TCF-2	8	38.61	38.4 - 38.8
TCF-3	9	38.48	38.2 - 38.7
C-1	10	38.32	38.0 - 38.7
C-2	11	38.42	38.0 - 38.8
C-3	12	38.47	38.1 - 38.7

Animal Species	Inoculum	Vol. (ml)	Route	No.	Lesions, Illness or Deaths over 4 to 6 Week Period	Comments
Adult Rabbits (1500-2500 gms)	Virus Pool Un-neutralized	10 x 0.1 1 x 9.0 1 x 0.03	I.D. S.Q. L.Cornea	2	There were no signs of illness or distress and no lesions at sites of inoculation.	Test Satisfactory
	Control Fluid (TCF)	10 x 0.1 1 x 9.0 1 x 0.03	I.D. S.Q. R. Cornea	2		
	None	——	——	1		

Table III - C. Animal Safety Tests Results on the Crude Japanese Encephalitis Vaccine Control and Virus Fluids

Animal Species	Inoculum	Vol. (ml)	Route	No.	Lesions, Illness or Deaths over 4 to 6 Week Period	Comments
Adult Mice (15-20 grams)	Virus Pool Un-neutralized	0.03 0.50	I. Cer. I.P.	20	No deaths nor signs of illness or distress recorded.	Test Satisfactory
	Virus Pool Neutralized	0.03 0.50	I. Cer. I.P.	20		
	Control Fluid (TCF)	0.03 0.50	I. Cer. I.P.	20		
	None	—	—	5		
Suckling Mice (< 24 hours)	Virus Pool Un-neutralized	0.01 0.10	I. Cer. I.P.	20	All 20 sucklings found cannibalized on day 8.	
	Virus Pool Neutralized	0.01 0.10	I. Cer. I.P.	20	1 (TCF) inoculated suckling found canniablized on day 14. No other deaths nor signs of illness or distress over this initial 14-day period.	
	Control Fluid (TCF)	0.01 0.10	I. Cer. I.P.	20		
	None	—	—	10		
	D14 Blind Passage (VP-N)	0.01 0.10	I. Cer. I.P.	20	3 of (VP-N) Blind Passage & 2 of (N) sucklings found cannibalized within first 24 hours. No other deaths nor signs of illness or distress over this final 14-day period.	Test Satisfactory 100% survival of inoculated sucklings. No evidence of a transmissible agent or of any viral infection.
	D14 Blind Passage (CF-TCF)	0.01 0.10	I. Cer. I.P.	20		
	D14 Blind Passage (N)	0.01 0.10	I. Cer. I.P.	10		
	D14 - None	—	—	10		

Table III - D. Animal Safety Tests Results on the Crude Japanese Encephalitis Control and Virus Fluids

Animal Species	Inoculum	Vol. (ml)	Route	No.	Lesions, Illness or Deaths over 4 to 6 Week Period	Comments
Adult Guinea Pigs (350-450 gms)	Virus Pool Un-neutralized	0.10 5.00	I. Cer. I.P.	3	No deaths nor signs of illness or distress. Daily rectal temperatures taken over last 3 weeks of observation were within normal ranges.	
	Virus Pool Neutralized	0.10 5.00	I. Cer. I.P.	3		
	Control Fluid (TCF)	0.10 5.00	I. Cer. I.P.	3		
	None	——	——	3		

Code	GP#	Mean Temp. (^{O}C)	Temp. Range (^{O}C)
VP-UN-1	0	38.40	38.0 - 38.7
VP-UN-2	1	38.53	38.1 - 38.8
VP-UN-3	2	38.35	38.0 - 38.7
VP-N-1	3	38.54	38.2 - 38.9
VP-N-2	4	38.25	37.9 - 38.5
VP-N-3	5	38.30	38.1 - 38.5
TCF-1	6	38.36	38.1 - 38.6
TCF-2	7	38.35	38.1 - 38.6
TCF-3	8	38.34	38.1 - 38.5
C-1	9	38.32	38.0 - 38.6
C-2	10	38.45	38.2 - 38.7
C-3	11	38.37	38.1 - 38.6

Adult Rabbits (1500-2500 gms)	Virus Pool Un-neutralized	10 x 0.1 1 x 9.0 1 x 0.03	I.D. S.Q. L.Cornea	2	There were no signs of illness or distress and no lesions at sites of inoculation.	Test Satisfactory
	Control Fluid (TCF)	10 x 0.1 1 x 9.0 1 x 0.03	I.D. S.Q. R. Cornea	2		
	None	——	——	1		

Table IV. General Safety Test Results on the Final Product of Japanese Encephalitis - Production Seed and Vaccine

Animal Species	Inoculum	Vol. (ml)	Tag #	Weight in Grams Day 0	Weight in Grams Day 7	Weight Gain/ (Loss) in Grams
Mice	Production Seed Virus	0.5	291	19.0	23.0	4.0
			292	18.2	24.1	5.9
	Vaccine	0.5	293	17.5	22.0	4.5
			294	18.0	22.0	4.0
	None	---	295	17.4	23.2	5.8
			296	18.1	23.8	5.7
Guinea Pigs	Production Seed Virus	5.0	5	334.0	420.5	86.5
			6	319.0	388.1	69.1
	Vaccine	5.0	3	335.0	447.0	112.0
			4	367.0	412.0	45.0
	None	---	9	409.0	456.0	47.0
			10	403.0	465.1	62.1

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Flow Laboratories, Inc.

A Flow General Company

June 24, 1987

Dr. Louis Potash
Flow Laboratories, Inc.
7655 Old Springhouse Road
McLean, Virginia 22102

Charge #833/8340

Dear Dr. Potash:

Your four samples, JBE pre-vaccine Virus, JBE pre-vaccine Control, JBE Vaccine Virus and JBE Vaccine Control submitted for the presence of mycoplasma hvorhinis using direct immunoflorescence staining, the DNA Hoechst stain and agar testing were found to be negative.

Sincerely,

Jim Quartey

Jim Quartey

JQ/sw

MYCOPLASMA TEST RECORD SHEET

Culture Medium	LOT #	No. ml Tested Aerobic	No. ml Tested Anaerobic	Date On Test	Date Off Test	Results
Virus Fluid - LOT # (PV JBE [illegible] -Vaccine Virus=MYC# 130						
PPLO Agar	870305	.2	.2	5/20/87	6/[illegible]	NEGATIVE
PPLO Broth	870406	25.0	25.0			NEGATIVE
D 5 Subpass to Broth		25.0	25.0	5/27/87	6/[illegible]	NEGATIVE
to Agar		.2	.2			NEGATIVE
D10 Subpass to Broth		25.0	25.0	6/[illegible]	6/[illegible]	NEGATIVE
to Agar		.2	.2			NEGATIVE
D15 Subpass to Broth		25.0	25.0	6/[illegible]	6/[illegible]	NEGATIVE
to Agar		.2	.2			NEGATIVE
Control Fluid - LOT # (PV JBE [illegible] -Vaccine Control=MYC# 131						
PPLO Agar		.2	.2	5/20/87	6/[illegible]	NEGATIVE
PPLO Broth		25.0	25.0			NEGATIVE
D 5 Subpass to Broth		25.0	25.0	5/[illegible]	6/[illegible]	NEGATIVE
to Agar		.2	.2			NEGATIVE
D10 Subpass to Broth		25.0	25.0	6/[illegible]	6/[illegible]	NEGATIVE
to Agar		.2	.2			NEGATIVE
D15 Subpass to Broth		25.0	25.0	6/9/87	6/[illegible]	NEGATIVE
to Agar	Y	.2	.2			NEGATIVE

Positive Control (+): [illegible] Negative Control (-): [illegible]

Date: 6/24/87 Signed: Vinz Culverley

MYCOPLASMA TEST RECORD SHEET

Culture Medium	LOT #	No. ml Tested		Date		Results
		Aerobic	Anaerobic	On Test	Off Test	
Virus Fluid - LOT # (VV) VBF-Vaccine Virus = Myc # 154						
PPLO Agar	276305	.2	.2	5/27/87	6/3/87	NEGATIVE
PPLO Broth	276406	25.0	25.0			NEGATIVE
D 5 Subpass to Broth		25.0	25.0	5/27/87	6/3/87	NEGATIVE
to Agar		.2	.2			NEGATIVE
D10 Subpass to Broth		25.0	25.0	6/3/87	6/8/87	NEGATIVE
to Agar		.2	.2			NEGATIVE
D15 Subpass to Broth		25.0	25.0	6/8/87	6/13/87	NEGATIVE
to Agar		.2	.2			NEGATIVE
Control Fluid - LOT # (VT) VBF-Vaccine Control Myc # V35						
PPLO Agar		.2	.2	5/27/87	6/3/87	NEGATIVE
PPLO Broth		25.0	25.0			NEGATIVE
D 5 Subpass to Broth		25.0	25.0	5/27/87	6/3/87	NEGATIVE
to Agar		.2	.2			NEGATIVE
D10 Subpass to Broth		25.0	25.0	6/3/87	6/8/87	NEGATIVE
to Agar		.2	.2			NEGATIVE
D15 Subpass to Broth		25.0	25.0	6/8/87	6/13/87	NEGATIVE
to Agar	↓	.2	.2			NEGATIVE

Positive Control (+): [illegible] Negative Control (-): [illegible]

Date: 6/24/87 Signed: [signature]

Main Office
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SPAFAS, Incorporated

Laboratory:
67 Baxter Road
Storrs, CT 06268
Tel: 203/429-1990
Tlx: 269965

QUALITY CONTROL SHEET

SPF-COFAL

Flock M950 **Hatch Date** 8/8/86 **No. In Flock** 4800

AGENT	ANTIGEN	TEST	100% Test	5% Test
AVIAN ADENO (CELO-PHELPS)	CELO-Phelps	AGP,SN,EIA	-	-
AVIAN ADENO II (EDS-76)	McFerran	HI	-	-
AVIAN ENCEPHALOMYELITIS	Van Roekel	FS,SN,AGP,EIA	-	-
AVIAN REOVIRUS	UC 1133,#25	AGP,MNT,EIA	-	-
FOWL POX	Conn.	AGP	-	-
HEMOPHILUS GALLINARUM (Coryza)		CLINICAL	-	-
HEMORRHAGIC ENTERITIS VIRUS	Domermuth	AGP	-	-
INFECTIOUS BRONCHITIS (Conn)	Conn 5968	SN,AGP,HI	-	-
INFECTIOUS BRONCHITIS (Mass)	Mass 66579	SN,AGP,HI,EIA	-	-
INFECTIOUS BURSAL DISEASE	M4040 (2512	AGP,SN,EIA	-	-
INFECTIOUS LARYNGOTRACHEITIS	UC A92430	SN,AGP	-	-
INFLUENZA (TYPE A)	T/M/66	AGP,SN	-	-
LYMPHOID LEUKOSIS VIRUS	GROUP A,B,C,D,E	COFAL,EIA	-	-
LYMPHOID LEUKOSIS ANTIBODY	RSV-RAV A	SN	-	-
LYMPHOID LEUKOSIS ANTIBODY	RSV-RAV B	SN	-	-
MYCOPLASMA GALLISEPTICUM	Commercial	SPA,HI,EIA	-	-
MYCOPLASMA SYNOVIAE	Commercial	SPA,HI,EIA	-	-
NEWCASTLE DISEASE	LaSota	HI,AGP,EIA	-	-
SAL. PULLORUM-TYPHOID	Commercial	SPA,IA,TA	-	-

PREVIOUS NEGATIVE 5% SAMPLING DATES	NEGATIVE WEEKLY TESTS FOR: AEV, LLV, REO, IBDV, and ADENO	
12/22/86	12/8/86	
1/19/87	12/15/86	
	12/22/86	
2/16/87	12/29/86	
	1/5/87	
3/16/87	1/12/87	
	1/19/87	
4/13/87	1/26/87	
	2/2/87	
	2/9/87	
	2/16/87	
	2/23/87	
	3/2/87	
	3/9/87	
	3/16/87	
	3/30/87	
	4/6/87	
	4/13/87	
	4/20/87	
	4/27/87	
	5/4/87	
	5/11/87	
	5/18/87	
	5/25/87	
	6/1/87	
	6/8/87	
	6/15/87	

AGP = Agar Gel Precipitin
COFAL = Complement Fixation Avian Leukosis
EIA = Enzyme Immunoassay
FS = Flock Susceptibility
HI = Hemagglutination Inhibition
IA = Isolation of Agent
MNT = Microneutralization
SN = Serum Neutralization
SPA = Serum Plate Agglutination
TA = Tube Agglutination

100% Test Completion Date 1/13/87 For 12/1/86 Sampling.
5% Test Completion Date 6/17/87 For 5/11/87 Sampling.

Laboratory Manager

Ben Venue Laboratories, Inc.
270 Northfield Road P.O. Box 46568 Bedford, Ohio 44146, 216-232-3320, Telex 810-427-2275, Ben Venue BDFD, Panafax 216-232-2772

Ben Venue LABORATORIES

July 22, 1987

Dr. Louis Potash
Flow Labs., Inc.
7655 Old Springhouse Rd.
McLean, VA 22101

Dear Dr. Potash,

Please be advised that the Sterility testing has been completed on the following materials, Under Flow P.O. #91901.

1) Japanese Encephalitis Virus Production Seed.
Lot #PDK8-WR2

2) Japanese Encephalitis Vaccine Live Attenuated Virus.
Lot #PDK9-WR3

Both lots were tested utilizing the membrane filtration method and incubation period of fourteen days. Each of the two lots were found to be sterile.

Copies of test sheets #S7096PF and #S7097PF are enclosed for your files.

With Kind Regards,
Ben Venue Laboratories, Inc.

Dorothy P. Dougherty
Manager, Microbiological Control

Encl.

CC: R. Haggerty

DPDcg

STERILITY TEST OF POWDERS
USP Membrane Filter Method

Date Sampled NA
Date Received 7-7-87
No. of Samples Received/Tested 18

BVL Control No. S 7 096 PF
Product Japanese Encephalitis Virus Production Seed
Lot No. PDK8-WR2
Thioglycollate No. L7118
Soybean-Casein Digest No. L7117
Date of Test 7-8-87
Operators Andrea M. McMurray
N/A

Sample Reconstituted with Sterile dist. H2O
Lot No. P057164
Reconstituted Volume 10 mL
Type of Membrane Filter Used 0.45 µm
Volume of Recon'd Sample Filtered 108 mL
Volume of Fluid Thioglycollate 100 mL
Volume of Soybean-Casein Digest 100 mL
Volume of 0.1% ~~Pep~~tone Wash 100 (P7154) mL

Test Time 0945 to 1130

	Thioglycollate	SCD
No. of Tubes used for Sterility Sample	1	1
No. of Reconstitution Fluid Controls	1	1
No. of Filter Controls	1	1
No. of Blank Media Controls	1	1
No. of Air Sham Media Controls	1	1
No. of 0.1% ~~Pep~~tone Wash Controls	1	1
No. of Tubes used for Water Control	NA	NA
No. of Tubes used for 250ml filter funnel controls	1	1

RESULTS:	Samples	Controls	Checked by
Date Read	7.22.87	7-22-87	C. Barber
Fluid Thioglycollate (Present/Absent)	Absent	Absent	
No. of Tubes Contaminated	0	0	
Date Read	7-22-87	7-22-87	C. Barber
Soybean-Casein Digest (Present/Absent)	Absent	Absent	
No. of Tubes Contaminated	0 MK 7-22-87 JAPANESE ENCEPHALITIS VIRUS	0	

On the basis of the above data, PRODUCTION SEED, BVL Lot No. PDK8-WR2
Customer Lot No. PDK8-WR2 is sterile and is acceptable
as of 7-22-87.
Identification: N/A

Carol Barber
Bacteriologist/Senior Technician

Madeline Kroggel
~~Manager~~, Microbiology Department
MK 7-22-87

COMMENTS: Steritest ®

New
Revised X
Replaces 12/16/81
Date 4/24/85

STERILITY TEST OF POWDERS
USP Membrane Filter Method

Date Sampled NA
Date Received 7-7-87
No. of Samples Received/Tested 2/2

BVL Control No. S 7 097 PF
Product Japanese Encephalitis Vaccine Live Attenuated Virus
Lot No. PDK9 - WR3
Thioglycollate No. L7118
Soybean-Casein Digest No. L7117
Date of Test 7-8-87
Operators Donna McMurray / N/A

Sample Reconstituted with sterile distilled H_2O
Lot No. BCS 714
Reconstituted Volume 10 mL
Type of Membrane Filter Used 1.2 um
Volume of Recon'd Sample Filtered 120 mL
Volume of Fluid Thioglycollate 100 mL
Volume of Soybean-Casein Digest 100 mL
Volume of 0.1% ~~Pep~~tone Wash 600 (P7154) mL

Test Time 0945 to 1130

	Thioglycollate	SCD
No. of Tubes used for Sterility Sample	1	1
No. of Reconstitution Fluid Controls	1	1
No. of Filter Controls	1	1
No. of Blank Media Controls	1	1
No. of Air Sham Media Controls	1	1
No. of 0.1% ~~Pep~~tone Wash Controls	1	1
No. of Tubes used for Water Control	NA	NA
No. of Tubes used for 250ml filter funnel controls	1	1

RESULTS:	Samples	Controls	Checked by
Date Read	7-22-87	7-22-87	C Barber
Fluid Thioglycollate (Present/Absent)	Absent	Absent	
No. of Tubes Contaminated	0	0	
Date Read	7-22-87	7-22-87	C Barber
Soybean-Casein Digest (Present/Absent)	Absent	Absent	
No. of Tubes Contaminated	0	0	

On the basis of the above data, ~~JAPANESE~~ VACCINE LIVE ATTENUATED ENCEPHALITIS VIRUS, BVL Lot No. PDK9 - WR3
Customer Lot No. PDK9 - WR3 is sterile and is acceptable
as of 7-22-87.
Identification: N/A

Carol Barber
Bacteriologist/Senior Technician

Manager, Microbiology Department
MR 7-22-87

COMMENTS: Sterilest®

New
Revised X
Replaces 12/16/81
Date 4/24/85

RESULTS OF REVERSE TRANSCRIPTASE ASSAY FOR STUDY 833

SAMPLE	rAdT Mg	rAdT Mn	dAdT Mn
1. JBE: Pre-vaccine virus pool	707	996	
2. JBE: Pre-vaccine TCF	785	1507	
3. JBE: Vaccine virus pool	1201	4144	93,745
4. JBE: Vaccine TCF	1191	4288	64,561
Controls:			
1. Growth medium	240	294	814
2. Medium from PR-RSV-A infected cells	419,149	275,011	
3. 40 µl medium + 10 µl medium from MMLV infected cells	942,092	2,140,548	

All reactions contained 50µl of sample.

Conclusions: None of the samples contain reverse transcriptase. Samples 3 and 4 contain high levels of DNA dependent DNA polymerase.

DISTRIBUTION LIST

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